IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Robert G. Pergolizzi et al.	)	
Serial No. 08/479,995	)	Group Art Unit: 1809
Filed: June 7, 1995	)	Examiner: Ardin H. Marschel, Ph.D
Title: **ANALYTE DETECTION UTILIZING POLYNUCLEOTIDE SEQUENCES, COMPOSITION, PROCESS AND KIT**	)	

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Honorable Commissioner of Patents and Trademarks
The United States Patent and Trademark Office
Washington, D.C. 20231

DECLARATION OF DR. JAMES G. WETMUR

I, James G. Wetmur, hereby declare as follows:

1. I am currently Professor of Microbiology and Human Genetics at the Mount Sinai School of Medicine in New York City, having held that position since 1994. I am also currently Professor of Microbiology at Mount Sinai, having held that position since 1983. During my sabbatical in 1992, I was Visiting Scientist at Roche Molecular Systems in Alameda, California. Previously from 1974 to 1982, I was Associate Professor of Microbiology at Mount Sinai. From 1969 to 1974, I was Assistant Professor of Chemistry and Biochemistry at the University of Illinois. I received my B.S. in chemistry from Yale University in 1963 and my Ph.D in chemistry from the California Institute of Technology in 1967. My thesis advisor was Dr. Norman Davidson. My professional experience and education are listed on my curriculum vitae (CV) attached to this Declaration as Exhibit 1.

2. My professional activities, honors and scientific publications, the latter numbering over eighty, are also listed in my CV (Exhibit 1). As seen in my CV, I have investigated nucleic acid hybridization reactions including the kinetics of DNA renaturation. I coauthored a 1968 paper with Dr. Davidson on the latter subject [Wetmur and Davidson, "Kinetics of renaturation of DNA," J. Mol. Biol. 31:349-370 (1968)]. In my investigation of nucleic acids, DNA hybridization and renaturation kinetics spanning some thirty years, I have examined nucleic acids including DNA from a number of different species using a number of different and diverse formats. I am thoroughly familiar with nucleic acid detection formats and nucleic acid probe technology, having spent the better part of my professional career exploring their use as investigative tools for nucleic acid hybridization and kinetic studies.

3. I have been asked by Enzo Biochem, Inc., for whom I have worked as a consultant from 1984 to 1991, to review portions of the current prosecution of U.S. Patent Application Serial No. 08/479,995 ("Analyte Detection Utilizing Polynucleotide Sequences, Composition, Process and Kit") that was filed on June 7, 1995. I am being compensated as a consultant by Enzo in connection with this matter. I have read the specification and pending claims in this application, as well as the two Office Actions issued on March 11, 1997 and October 27, 1997, and Enzo's July 25, 1997 Amendment Under 37 C.F.R. §1.115. I am familiar with the publication, Dunn and Hassell ["A Novel Method to Map Transcripts: Evidence for Homology between an Adenovirus mRNA and Discrete Multiple Regions of the Viral Genome," Cell 12:23-36 (1977)] that the Patent Examiner cited for anticipation against the pending claims in both Office Actions. A copy of the cited Dunn and Hassell (1977) publication is attached to this Declaration as Exhibit 2.

4. I understand that in the March 11, 1997 Office Action, the Examiner's position on anticipation was as follows:

> Dunn et al. reads on the above listed claims due to its disclosure of an immobilized target analyte wherein a bridging entity is hybridized thereto followed by washing and then the hybridization of a nick translated radiolabelled signalling entity that is made up of a heterogeneous mixture of radiolabelled fragments produced as a result of the nick translation process. This nick translation process also results in a ratio of signalling entities as being clearly greater than 1 as compared to bridging entities, but is unclear how much greater than 1.

I have also reviewed the anticipation rejection that was maintained in the October 27, 1997 Office Action. A copy of the text of the Examiner's remarks with respect

to the anticipation rejection is attached as Exhibit 3.

5. I have reviewed and understand that claims 283-362, 364-380, 382-398, 400-404, 406-439 and 441-462 are pending in this application. A copy of claims 283-294, which are representative of the pending composition claims, is attached as Exhibit 4. I also understand that other pending claims in this application are directed to processes and kits for detecting an analyte having one or more molecularly recognizable portions thereon. All such processes and kits rely on the use or inclusion of the pending composition claims (Exhibit 4).

6. Sandwich hybridization as first disclosed by Dunn and Hassell (1977) requires the use of two nucleic acid probes, one nucleic acid being an immobilized capture probe and the other nucleic acid being a radioactively labeled signaling probe. In sandwich hybridization, the capture probe and the signaling probe are complementary to adjacent nonoverlapping sequences in the target nucleic acid. The capture probe and the signaling probe in sandwich hybridization and in Dunn's own cited disclosure meet the classical and art-accepted definitions and meaning of a nucleic acid probe. A nucleic acid probe of known sequence interacts with a corresponding target nucleic acid for the purpose of determining its presence. That a nucleic acid probe interacts with its yet to be identified target analyte is a familiar and well-recognized scientific concept that has existed for many years.

A. On the very first page of the well-known and well-accepted texts on DNA probes [aptly titled DNA Probes, Keller and Manak, Stockton Press, New York, 1989, Chapter 1, and DNA Probes: Background, Applications, Procedures, Second Edition, Keller and Manak, Stockton Press, New York, 1993, Chapter 1], George H. Keller defines "probe" as:

> **THE PROBE-TARGET INTERACTION**
>
> ***A probe in the chemical or biological sense, is a molecule having a strong interaction only with a specific target and having a means of being detected following the interaction***. Examples of such strong and specific probe-target interactions are: antibody-antigen, lectin-carbohydrate, avidin-biotin, receptor-nucleic acid and interactions between complementary nucleic acids. Protein probes (i.e., antibodies) interact with their specific target through a mixture of forces: hydrophobic, ionic and hydrogen bonding, at only a few specific sites. By contrast, **nucleic acid probes interact with their complement** primarily through H-bonding, at tens, hundreds or thousands of sites, depending on the length of the hybrid. Hydrophobic interactions also play a role, as evidenced by the

reduction of hybrid stability by organic solvents, but probably contribute little to specificity. [bold & underline added]

A copy of page 1 from each of Keller and Manak's first and second editions of DNA Probes is attached as Exhibit 5.

B. In their introductory section and chapter to the book Nucleic Acid Probes [Robert H. Symons, Editor, CRC Press, Inc., Boca Raton, Florida, 1989, Chapter 1, "Enzymatic and Chemical Techniques for Labeling Nucleic Acids with Radioisotopes," page 2], McInnes and Symons provide the following description for nucleic acid probes:

> The basis for studies involving **nucleic acid probes** is the technique known as nucleic acid hybridization analysis. Briefly, the **nucleic acid probe**, either labeled with a radioactive isotope (this chapter) or nonisotopically (Chapter 2), **hybridizes to form a duplex *only with target nucleic acid*** (e.g. DNA or RNA) which is exactly complementary to itself; no such double-strand hybrid is formed with other nucleic acids. [bold & underline added]

A copy of page 2 from Symons' Nucleic Acid Probes is attached as Exhibit 6.

C (i). In the opening chapter to their book on probes for clinical pathologists, Nucleic Acid Probes: A Primer for Pathologists [American Society of Clinical Pathologists, ASCP Press, 1989, Chapter 1, "Nucleic acid chemistry and cell biology," page 26], Margaret A. Piper and Elizabeth R. Unger provide the following definition:

> **Nucleic Acid Probes**
>
> A **nucleic acid probe** is a well-characterized, relatively short sequence of nucleotides that can anneal with complementary sequences in **test [analyte] nucleic acid molecules**. . . [bold & parenthetical added]

C (ii). In their glossary (page 136), Piper and Unger also provide the following definition for probe:

> A ***known labeled sequence of DNA or RNA*** **used to detect** any complementary sequences in the **target** polynucleotides after specific hybridization. [bold & underline added]

A copy of pages 26 and 36 from Piper and Unger's primer is attached as Exhibit 7.

D. Other classical definitions for probes have been provided in the literature:

> **probe** . . . A substance, frequently labeled with radioactive isotopes, that is used to identify or isolate a gene, a gene product, or a protein. The hybridization of mRNA with its DNA gene, the hybridization of chromosomal DNA with corresponding cDNA fragments, and the binding of specific protein molecules to monoclonal antibodies are some examples of the use of probes. . .

From the Dictionary of Biochemistry and Molecular Biology, Second Edition, J. Stenesh, John Wiley & Sons, New York, 1989, page 382, copy attached as Exhibit 8.

> **probe** As a noun, a probe is a specific DNA or RNA sequence which has been radioactively labelled to a high specific activity. **Probes are used to detect complementary sequences** by hybridisation techniques such as Southern or Northern blotting or colony hybridisation. As a verb, 'to probe' is the act of hybridisation to detect a specific gene or transcript. E.g. 'We probed our bank with labelled rRNA to detect clones containing rDNA sequences.' [bold & underline added]

From A Dictionary of Genetic Engineering, Stephen G. Oliver and John M. Ward, Cambridge University Press, Cambridge, 1985, page 83, copy attached as Exhibit 9.

7. First disclosed by Dunn and Hassell (1977), the sandwich hybridization technique was directed toward overcoming the problem of non-specific binding of non-analyte sequences to capture sequences. Non-specific binding limited the specificity of analyte detection. By using a signaling sequence complementary to a portion of the nucleic acid analyte, an additional level of specificity was introduced by the sandwich hybridization technique: The signaling sequence hybridized to the bound nucleic acid sequence in preference to non-specifically bound non-analyte sequences.

A. The RNA target analyte in Dunn and Hassell (1977) comprised both adenovirus RNA containing SV40 sequences and adenovirus RNA lacking SV40 sequences as well as cellular messenger RNAs also lacking SV40 sequences. Each of the capture sequences immobilized on their filter was capable of binding specifically to a subset of both types of adenovirus RNA and non-specifically to the remaining adenoviral and cellular RNAs. Only adenovirus RNAs that also contained SV40 sequences were able to hybridize to the signaling sequence, specifically identifying these particular messages that were of interest to Dunn and Hassell.

B. The present invention does not fulfill the accepted criteria for "sandwich hybridization" because it does not increase the specificity of analyte detection by providing two analyte-specific probes as in the case of Dunn and Hassell (1977). Among other objects and features, the present invention discloses unique means and compositions for extending the range of applicability of expensive-to-produce signaling sequences wherein the signaling moiety is attached to an arbitrary nucleic acid sequence not found in the analyte of interest. The specificity for linking any signaling sequence to any particular analyte is mediated through a linker nucleic acid sequence containing one portion complementary to the analyte nucleic acid and a second portion complementary to the arbitrary nucleic acid sequence placed in the signaling molecule.

C. In further detail, as required for sandwich hybridization and as first disclosed in their cited paper, Dunn and Hassell (1977) used in their study two nucleic acid probes - one a capture probe and the other a signaling probe - to map target analytes in the form of viral RNA transcripts. Dunn's capture probe was adenovirus type 2 DNA immobilized to a nitrocellulose filter and their signaling probe was ^{32}P-labeled SV40 DNA. Both of Dunn's probes - the capture probe and the signaling probe - were complementary to different portions of the target RNA transcript analyte being mapped. The foregoing characterization is confirmed by Dunn and Hassell's own description in their 1977 paper:

> An outline of the procedure we have used to map viral RNAs comprised of both SV40 and Ad2 sequences is shown in Figure 1B. Ad2 DNA is cleaved with a restriction endonuclease, the resulting fragments separated by agarose gel electrophoresis and transferred to a nitrocellulose filter (Southern, 1975). **Unlabeled RNA [analyte] extracted from cells** lytically infected with an adenovirus-SV40 hybrid is hybridized to the immobilized DNA fragments, the filters are washed to remove unannealed RNA and hybridization is continued with SV40 DNA that has been labeled in vitro with ^{32}P to high specific activity (citations omitted). After again washing the filters, the Ad2 DNA fragments complementary to RNA which contains SV40 sequences can be identified by autoradiography. We have in this way **demonstrated the existence of an RNA molecule consisting of Ad2 and SV40 sequences in one continuous chain** and have mapped the location of the sequences which serve as template for its synthesis on the Ad2+ND1 genome.

[Dunn and Hassell, page 23, right column; bold, underline & parenthetical added]

8. I further conclude from my review of Dunn and Hassell (1977) that their target analyte was and could only have been the RNA transcripts that the authors were seeking to map. This conclusion was reached because the authors had

prepared their capture probe by isolating the adenovirus type 2 DNA and immobilizing it onto a nitrocellulose filter, and they had prepared their signaling probe by labeling SV40 DNA with hot phosphorus (^{32}P). Therefore, the target analyte in Dunn and Hassell (1977) had to be the only remaining entity in their disclosure, namely, the RNA transcript that became sandwiched between the immobilized capture probe and the signaling probe. This is the only characterization that fits the sandwich hybridization technique as disclosed by Dunn and Hassell (1977) and as later described in the scientific literature. In addition to that portion from Dunn and Hassell (1977) quoted in the preceding paragraph, my conclusion regarding the RNA transcript being the analyte in their disclosure is supported by Dunn's own Figure 1B on page 24, specifically, the lower right portion, which provides an illustration of their sandwich hybridization technique. Dunn's illustration clearly depicts the target RNA transcript analyte sandwiched between the capture probe (immobilized Ad2 DNA) and the signaling probe (SV40 ^{32}P-labeled DNA).

9. My conclusion set forth in the preceding paragraph that Dunn's analyte was the RNA transcript is also based on the fact that the authors were mapping RNA transcripts. This is acknowledged even by the Examiner in the October 27, 1997 Office Action (page 7, lines 16-17) where he states: "since the Dunn et al. disclosure is directed to mapping RNA sequences, this is deemed to be the target." Support for RNA transcript mapping is shown by several portions in Dunn and Hassell (1977) referred to below:

- The very title of Dunn's article is "*A Novel Method to Map Transcripts*: Evidence for Homology between an Adenovirus mRNA and Discrete Multiple Regions of the Viral Genome." [underline added]

- "A method has been devised which permits mapping of transcripts by a two-step hybridization procedure (sandwich hybridization)." [Summary, page 23, underline added]

- "Several approaches to mapping the regions of adenovirus type 2 (Ad2) DNA which are transcribed have been developed in recent years." [Introduction, page 23, underline added]

- "By using DNA radioactively labeled to high specific activity in vitro as a probe, we have overcome some of the limitations to mapping RNA labeled in vivo." [Introduction, page 23, underline added]

10. I find it of some significance that Dunn and Hassell (1977) were the first to describe the sandwich hybridization technique and that the literature generally accords them recognition for having done so. The scientific literature published after Dunn and Hassell (1977) supports my conclusion that Dunn's target analyte was the RNA transcript sandwiched between their immobilized capture probe and their isotopically labeled signaling probe.

A. For example, in both the first and second editions of DNA Probes, cited *supra*, Keller and Manak credit Dunn and Hassell with first describing nucleic acid sandwich hybridization while describing the technique and its advantages:

> The sandwich hybridization format was originally described by **Dunn and Hassell (1977)** and adapted by Ranki *et al.* (1983) and Ranki and Soderlund (1984). It was developed to avoid the tedious purification and immobilization of sample nucleic acid required in most solid phase hybridization formats. Sandwich hybridization has two main advantages over direct filter hybridization, **sample [analyte] immobilization is not required** and crude samples can be assayed reliably. In addition, sandwich hybridization is potentially more specific than direct hybridization because two hybridization events must occur in order to generate a signal. Solid phase sandwich hybridization requires two adjacent, non-overlapping probes: **an immobilized capture probe and a labeled detection probe**. Figure 5.8 illustrates a typical sandwich hybridization scheme consisting of an **immobilized capture sequence** cloned into M13 and **an adjacent detection sequence** cloned into pBR322. A sandwich structure can form only if the sample contains nucleic acid which spans the original junction between the two fragments in genomic nucleic acid. . .
>
> [bold & underline added]

From DNA Probes, cited *supra*, Section Five: *Hybridization Formats and Detection Procedures*, pages 198-199; and DNA Probes: Background, Applications, Procedures, cited *supra*, Section Six, *Hybridization Formats and Detection* Procedures, pages 238-239, copy of both attached as Exhibit 10.

B. In a chapter twice published in a book dealing with nonisotopic DNA probes and probing, Peter Verlander of Rockefeller University similarly cites Dunn and Hassell when describing sandwich hybridization:

> **2. Sandwich Hybridizations**
>
> Sandwich assays are particularly well suited to peroxidase detection. These assays **generally** include a capture probe that is

> **bound to a fixed matrix, such as nitrocellulose membranes (Dunn and Hassell, 1977**; Ranki *et al.*, 1983), microtiter wells (Dahlen *et al.*, 1987; Keller *et al.*, 1989), or beads (Langdale and Malcolm, 1985; Polsky-Cynkin, *et al.*, 1985). **The target nucleic acid molecule hybridizes to the capture probe and is thereby bound to the matrix, while a second probe that is directly or indirectly labeled with peroxidase hybridizes to an adjacent sequence on the target** . . .
> [bold & underline added]

From Nonisotopic DNA Probe Techniques [Larry J. Kricka, editor, Academic Press, San Diego and New York, 1992, Chapter 8, "Detection of Horseradish Peroxidase by Colorimetry, pages 187-188], and Nonisotopic Probing, Blotting, and Sequencing [Larry J. Kricka, editor, Academic Press, San Diego and New York, 1995, Chapter 9, "Detection of Horseradish Peroxidase by Colorimetry," page 220], copy of both attached as Exhibit 11.

C. A predecessor review paper to Kricka's first book was published in 1988. In a section of that review paper titled "Solid Supports," Kricka and his coauthor provide another significant account of sandwich hybridization:

> Although simple and fairly rapid procedures, these methods still require the immobilization of individual sample DNAs. The **sandwich hybridization technique** described below allows for the large-scale preparation of an activated solid support coated with probe (Fig. 8). The **immobilized probe is used to collect the target DNA**, which is **then detected** ***using a labeled second probe (which does not itself interact with the immobilized probe***). [bold, underline & italic added]

From Mathews and Kricka, "Review: Analytical Strategies for the Use of DNA Probes," Analytical Biochemistry 169:1-25 (1988); copy attached as Exhibit 12; see pages 14 and 15.

D. Tijssen has also recognized Dunn and Hassell (1977) for their original description of the "capture hybridization method." In a volume published in the series Laboratory Techniques in Biochemistry and Molecular, the author writes:

> A compromise between the mixed phase and solution hybridization formats is the capture hybridization method originally described by Dunn and Hassell (1977) and adapted by Ranki et al. (1983). Target immobilization in standard solid phase hybridization has the disadvantage that many nonspecific molecules can be present and out-compete the target molecules for the binding sites available on the solid phase and thus influence the amount of target nucleic acid which can be detected. Although in some cases these competing macromolecules can be selectively removed (Verbeek et al., 1990), it makes the method laborious. Moreover, hybridization can be very slow at lower target nucleic acid concentrations on the membrane. These problems often can be avoided by using a **capture probe immobilized on the solid phase. The labeled probe hybridizes with high efficiency and rapidly to the target in solution and the hybrid is efficiently captured on the solid phase due to the high concentration of capture probe**. . . [bold & underline added]

From Hybridization With Nucleic Acid Probes, Part II: Probe labeling and hybridization techniques, P. Tijssen, published in Laboratory Techniques in Biochemistry and Molecular, Elsevier Science Publishers B.V., 1993, volume 24, Chapter 8, page 419, copy attached as Exhibit 13.

E. In a chapter published in Nucleic Acid Probes, cited *supra*, McInnes and Symons correctly describe the two-phase sandwich hybridization while incompletely attributing its development only to Ranki et al. ["Sandwich hybridization as a convenient method for the detection of nucleic acids in crude samples," Gene 21:77 (1983)]:

> **a. Two-Phase Sandwich Hybridization**
> . . . The general principle of the method is outlined in Figure 5A using nonradioactive single-stranded DNA probes in the phage M13 DNA as an example. **Two probes are required which hybridize to different, nonoverlapping regions of the target nucleic acid. One probe is bound to a solid support** such as nitrocellulose by standard procedures while the other is labeled with biotin using, for example, Photobiotin (Chapter 2, Section III.A.2). **The probe-bound filter is hybridized with test nucleic acid sample in the presence of the second biotin-labeled probe which can only be bound to the filter via the bridge of *target* nucleic acid**. After the usual washing procedures, the biotin-labeled probe is detected by standard procedures . . .
> [bold, italics & underline added]

See pages 130 and 131 from Nucleic Acid Probes, copy attached as Exhibit 14.

F. In Nucleic Acid Probes: A Primer for Pathologists, cited *supra*, Piper and Unger provide the following description of sandwich hybridization:

> The sandwich hybridization assay (Figure 2-3) is a modification of the dot/blot hybridization assay and was designed to overcome the problem of nonspecific signal generated with crude samples. The assay requires the purification of two nucleic acid reagents. These sequences are adjacent to one another in the target of interest, but are noncomplementary. **One sequence, known as the "filter" or "target" probe, is immobilized on the membrane. The other sequence is the labeled, or detection, probe**. The crude sample is interacted with the filter and detection probes simultaneously. **Only samples with sequences capable of forming a "sandwich" between the target and probe sequences will generate a signal**. . . [bold & underline added]

From Nucleic Acid Probes: A Primer for Pathologists, Chapter 2, Nucleic acid hybridization analyses and other nucleic acid assays, pages 49-50, copy attached as Exhibit 15.

G. Scientific investigators for the present Assignee have also described sandwich hybridization in accordance with the rest of the scientific literature. In the opening chapter to a book on Gene Probes for Bacteria, Goltz et al. provided the following description:

> Sandwich hybridization assays are extremely specific in that they require that at least **two different pathogen-specific probes hybridize to the target DNA, rather than just one.** In these assays, **one probe (the capture sequence) is bound to a solid support and is allowed to bind (capture) specimen DNA.** A second probe **(the signaling probe), with a sequence that is adjacent or close to the capture sequence on the target DNA is then allowed to hybridize to the support-bound target DNA.** This signaling probe can be labeled with either radioactive (^{3}H, ^{35}S, ^{32}P, ^{125}I) or nonradioactive molecules (biotin, sulfonates, digoxigenin, or fluorescent dyes).
> [bold & underline added]

From Gene Probes for Bacteria, Alberto J. L. Macario and Everly Conway de Macario, editors, Academic Press, Inc., San Diego and New York, 1990, Chapter 1, Goltz et al., "The Use of Nonradioactive DNA Probes for Rapid Diagnosis of Sexually Transmitted Bacterial Infections," pages 1-44; see page 6, copy attached as Exhibit 16.

H. More recently, Asim has provided the following description of sandwich hybridization published in the opening chapter of Nucleic Acid Analysis: Principles and Bioapplications [Charles A. Dangler, Editor, Wiley-Liss, New York, 1996, Chapter 1, "Nucleic Acid Hybridizations: Principles and Strategies," pages 12-13]:

> *Sandwich Hybridization.* Sandwich hybridization was developed to overcome the problem of nonspecific hybridization signals generated by the other conventional hybridization methods. Another advantage of this method is that the sample processing is minimum and thus the total time is enormously reduced. **A portion of the semipurified test DNA is hybridized with a target probe that was previously immobilized onto a solid support. Following hybridization, the rest of the "free" test DNA is subjected to hybridization with a probe DNA that is labeled either with radionucleotide or by colorimetric reagent.** After appropriate washing of the nonspecifically bound probe, the detection is performed through autoradiography or by colorimetric result. **This hybridization approach will only generate positive signal if there is a "sandwich" between the immobilized target probe and the labeled probe** (see Fig. 1.1B) . . . [bold & underline added]

See pages 9 and 12-13 from Nucleic Acid Analysis, copy attached as Exhibit 17.

I. Definitions for sandwich hybridization have also been provided by scientific and technical dictionaries. For example, in Gunter Kahl's Dictionary of Gene Technology, the following definition for sandwich hybridization is provided:

> **Sandwich hybridization:** **A method to identify a specific DNA sequence with two → probes which are homologous to different parts of the target DNA**. In short, **one probe ("catcher") serves to anchor the target DNA at a solid phase** (e.g. a microtiter plate, plastic-coated support, or chemically activated paper). Anchoring may be achieved by baking or by specific interactions (e.g. the affinity between solid phase-bound → streptavidin and → biotin that is incorporated in the catcher probe). **Then a second probe ("detector", reporter) is added that recognizes other regions of the target DNA and serves to detect the sandwich complex**, e.g. via an enzyme reaction that leads to a colored product at the sites of hybridization . . .
>
> [bold & underline added]

From Dictionary of Gene Technology, Gunter Kahl, VCH Publishers, Inc., New York, 1995, page 434, copy attached as Exhibit 18.

11. Among the literature, I can even point to Dunn's own group and coauthors as support for my conclusion that the RNA transcripts in Dunn and Hassell (1977) were the target analyte.

A. For example, in 1978, the lead author Dunn characterized their 1977 work in precisely the same way as it was first disclosed a year earlier, namely, as a sandwich hybridization technique for RNA transcript mapping:

> . . . The first of these, sandwich hybridization (Dunn and Hassell, 1977), depends upon the fact that of the late RNAs present, only the hybrid RNAs contain both SV40 and adenoviral sequences. Thus, **when the hybrid mRNAs (or a cDNA copy of them) are hybridized to defined fragments of adenoviral DNA bound to nitrocellulose filters**, they form duplexes through their adenoviral sequences, leaving their SV40 sequences as protruding tails. The process of annealing with ^{32}P-labeled SV40 DNA causes these tails to become labeled, permitting autoradiographic identification of the sequences of adenovirus 2 which are homologous to the hybrid mRNA. [bold & underline added]

From Dunn et al., "A Supplementary Adenoviral Leader Sequence and Its Role in Messenger Translation," Cell 15:511-526 (1978); emphasis added; copy attached as Exhibit 19.

B. Two years later In 1980, the lead author Dunn again characterized their 1977 paper in the same identical way, that is, the use of sandwich hybridization to map viral RNA transcripts:

> Messenger RNAs are most commonly assigned to specific genomic locations by demonstrating that they are complementary to a restriction fragment of DNA whose position within the genome is known. Hybridization to two adjacent fragments is usually taken to mean that a particular mRNA contains sequences which are contiguous within the genome and which span the restriction endonuclease cleavage site. However, this conclusion is fragile because the possibility exists that the mRNA preparation contains two or more species which happen to be complementary to adjacent genomic fragments. In eukaryotic systems, the interpretation is clouded because mature mRNAs generally are spliced and consist of sequences derived from noncontiguous genomic regions. The technique of **sandwich hybridization**[1] eliminates some of these problems, and **it provides a biochemical method to determine whether sequences from different regions of a genome are covalently joined to one another in mRNA.**
>
> In theory, the technique is of general application and can be used to analyze the transcription products of any segment of DNA whose restriction maps are known. . . [bold added]
>
> [1] A. R. Dunn and J. A. Hassell, *Cell* **12**, 23-36 (1977).

From Dunn and Sambrook, "Mapping Viral mRNAs by Sandwich Hybridization," Methods in Enzymology 65:468-478 (1980); copy attached as Exhibit 20.

12. As set forth in the paragraphs that now follow, it is my opinion and conclusion that the present invention and its elements represented by the pending claims in this application are different in at least two material elements from any composition or method disclosed in Dunn and Hassell (1977).

A. The first material claim element that is lacking in Dunn and Hassell (1977) is the presently claimed signaling entities that are capable of binding to or hybridizing with one or more molecular bridging entities which are distinct from the target analyte. In sandwich hybridization as disclosed in Dunn and Hassell (1977), both the capture probe (immobilized adenovirus type 2 DNA) and the the signaling probe (^{32}P-labeled SV40 DNA) are complementary to the target RNA transcript analyte. Thus, Dunn's capture and signaling probes in the sandwich hybridization technique hybridize to different portions of the target RNA transcript analyte. In contrast to Dunn's disclosure, the present invention specifically recites in the pending claims a requirement that the molecular bridging entity and the signaling entity be capable of binding to or hybridizing with each other through their respective nucleic acid portions. Thus, the present invention including the claimed compositions is materially different from any disclosed or used in Dunn and Hassell (1977).

B. A second material element missing altogether in Dunn and Hassell (1977) is the requirement in the present claims that the signaling entity or entities be substantially incapable of binding to or hybridizing with the analyte. It is my opinion and conclusion that this limitation in the claims in and of itself materially distinguishes the present invention from the sandwich hybridization technique disclosed in Dunn and Hassell (1977). What is disclosed in Dunn's paper is a sandwich hybridization technique that involves mapping target RNA transcript analytes. In the sandwich hybridization technique employed by Dunn and Hassell (1977), the target analyte (viral RNA transcripts) is sandwiched between the capture probe (immobilized adenovirus DNA type 2), and the signaling probe (radioactively labeled ^{32}P fragment of SV40 DNA). The signaling probe hybridizes to SV40 sequences found in Dunn's target RNA transcript analyte - and those SV40 sequences are not present in Dunn's capture probe. Because the signaling entities are incapable of interacting with the analyte, the present invention eschews sandwich hybridization altogether. This makes the present invention and claimed compositions materially different from Dunn and Hassell (1977).

13. It is my opinion and conclusion that several of the Examiner's assertions in the latest anticipation rejection set forth in the October 27, 1997 Office Action, including his characterization of Dunn and Hassell (1977) and the sandwich hybridization technique, are incorrect. In particular, I respectfully submit that certain erroneous references to what is the analyte in Dunn and Hassell (1977) are inconsistent and run altogether contrary to the authors' own disclosures and the scientific literature.

A. First, as set forth in Paragraphs 10A, 10B and 10D above, Dunn and Hassell (1977) were probably the first to disclose the use of the sandwich hybridization technique which in their disclosure was confined to isotopic labelling. For the two decades since Dunn's disclosure, the scientific literature has consistently described sandwich hybridization as requiring and employing two nucleic acid probes - an immobilized capture probe and a signaling probe. See Paragraphs 10 and 10A through 10I (ten "eye") above. The two probes hybridize to two separate and distinct portions of the target nucleic acid analyte (which in the case of Dunn and Hassell (1977) is the target RNA transcript analyte). Dunn's signaling probe hybridized to sequences in the target RNA transcript analyte, that target analyte being sandwiched between the immobilized capture probe and the signaling probe.

A (i) I respectfully submit that it was an error when the Examiner stated in the October 27, 1997 Office Action:

> Applicants firstly argue that the Dunn et al. disclosure utilizes sandwich hybridization wherein the two reagents entities hybridize to two separate portions of the target. **[1]**This is entirely contrary to the Dunn et al. reagents wherein there is an entity that hybridizes to the target RNA said entity having a tail. **[2]**The nick translated signal entities hybridize to said tail and not to the target RNA thus making this argument of applicants moot as directed to a mischaracterization of the disclosure of Dunn et al. [parenthetical numbers added]

A (ii) With respect to the assertions designated **[1]** and **[2]**, Dunn and Hassell (1977) specifically disclose in their opening Summary (page 23) the following series of events in their sandwich hybridization technique:

> RNA extracted from cells infected with an adenovirus-SV40 hybrid (Ad2+ND1) **[target analyte]** was hybridized to restriction endonuclease fragments of adenovirus type 2 (Ad2) DNA immobilized on nitrocellulose filters **[capture probe]**. RNA containing both Ad2 and SV40 sequences formed duplexes through their Ad2 sequences leaving their SV40 sequences as protruding tails.
>
> Annealing with ^{32}P-labeled SV40 DNA **[signaling probe]** caused these tails **[of the target analyte]** to become labeled . . ."
> [bold parentheticals added].

In Dunn's cited disclosure, therefore, it was their radioactively labeled signaling probe that hybridized to an SV40 tail sequence present in their ***target RNA transcript analyte***. Clearly, Dunn's immobilized capture probe contained no such tail sequence. Furthermore, to assert that in Dunn and Hassell (1977) there is an "entity that hybridizes to the target RNA said entity having a tail," would require one to accept wrongly that Dunn's analyte was the immobilized capture probe. It is my opinion that such a wrong acceptance would refute Dunn's own disclosure as well as two decades of scientific explanations of sandwich hybridization, including the cited authors' subsequent publications on the subject. Moreover, in my opinion, that acceptance would require one to discard the very clear and fundamental distinction that has been recognized and accepted over the years between a probe and its target analyte.

B. Second, as explained above in Paragraphs 7, 12B and 13A(ii), Dunn's ^{32}P-labeled SV40 DNA signaling probe hybridized only to the target RNA transcript analyte. The pending claims require that the signaling entity or entities be

substantially incapable of binding to or hybridizing with the analyte. I also believe that the following statements by the Examiner were in error:

> The second argument is that the signalling entities be substantially incapable of hybridizing to the molecularly recognizable portion on the analyte. **[1]**This argument is also non-persuasive because the Dunn et al. disclosure labels the target only via a sandwich formed when one reagent hybridizes to the target thus making it an incapability for the signalling entity to hybridize thereto thus inherently meeting the instant claim limitations.
>
> **[2]**Another element of support for the substantial incapability of the signalling entity to hybridize to the target recognizable portion is that these are distinct sequences in that one is Adenovirus type 2 sequence and the other is SV40 sequence. **[3]**These are well known to be different and distinct sequences thus being substantially incapable of hybridization as required for the instant claims and also supporting the rejection. [bold parenthetical added]

B (i) With respect to **[1]**, I respectfully submit that this assertion is incorrect because Dunn's signaling probe (^{32}P-labeled SV40 DNA) is clearly disclosed as hybridizing to their target RNA transcript analyte through the protruding SV40 tail sequences present in the target analyte. This has already been established by Dunn's own disclosure and enormous subsequent scientific literature dealing with nucleic acid probes and nucleic acid hybridization formats, including sandwich hybridization techniques. As particularly disclosed in Kricka's 1988 review article, cited *supra*:

> The sandwich hybridization technique described below allows for the large-scale preparation of an activated solid support coated with **probe DNA** (Fig. 8). The **immobilized probe is used to collect the target DNA**, which is **then detected using a labeled second probe** (**which *does not itself interact with the immobilized probe***).
>
> [bold, underline & italic added]

From Mathews and Kricka, *supra*, pages 14-15 (Exhibit 12).

B (ii) With respect to **[2]** and **[3]**, I respectfully submit that both assertions are incorrect because Dunn's signaling probe (^{32}P-labeled SV40 DNA) hybridized to the protruding SV40 tail sequences in Dunn's target RNA transcript analyte. Thus, Dunn's signaling probe must have possessed by that very fact a substantial capability for hybridizing to the target RNA transcript analyte. Accordingly, I must conclude that Dunn's isotopically labeled signaling probe does not meet the limitation in the pending claims that the signaling entity or entities be substantially

incapable of binding to or hybridizing with the molecularly recognizable portion on the analyte.

C. I understand that in its July 25, 1997 Amendment, Enzo had argued a distinction on the basis that Dunn's analyte RNA molecule consisted of adenovirus and SV40 sequences in one continuous chain, quoting Dunn and Hassell (1977):

> . . . We have in this way **demonstrated the existence of an RNA molecule consisting of Ad2 and SV40 sequences *in one continuous chain*** and have mapped the location of the sequences which serve as template for its synthesis on the Ad2+ND1 genome. . .
> [Dunn et al., Cell 12:23 (1977); left column, last paragraph; emphasis added]

Following the quote above, in its next statement in its July 25th Amendment, Enzo stated that "the analyte in the present invention could never have in one continuous chain the complementary sequences corresponding to both the molecular bridging entity and the signaling entity for the very clear and simple fact that the latter is substantially incapable of binding to or hybridizing with the analyte." I wholly agree with Enzo's statements regarding the distinction based upon the continuous chain of complementary sequences in Dunn's analyte. In Dunn's case, the target RNA transcript analyte had adenoviral sequences that hybridized to the immobilized capture probe (adenovirus DNA) and SV40 sequences in its protruding tails that hybridized to the signaling probe (^{32}P-labeled SV40 DNA). The adenovirus and SV40 sequences in Dunn's target RNA transcript analyte lay in one continuous chain, unlike the present invention where such sequences could not so lie in the analyte.

D. Based on my review of Dunn and Hassell (1977) and Ranki et al., U.S. Patent No. 4,486,539 (copy attached as Exhibit 19), I have also concluded that as related sandwich hybridization techniques, both disclosures require the use of two nucleic acid probes having complementary sequences to nonoverlapping sequences in their respective target analytes. The target analyte in both disclosures is further "sandwiched" between their respective sets of two probes, unlike the present invention in which the analyte is not sandwiched between the molecular bridging and signaling entities. In the October 27, 1997 Office Action, the Examiner stated the following:

> Applicants then summarize several references that refer to sandwich hybridization and credit various authors for suggesting or disclosing them. **[1]**It is acknowledged that sandwich hybridization is a phrase that may be applied to both the Ranki et al. type of assay as well as the Dunn et al. type of assay but that the location of the entity that is the target is different but that there are similarities in the sandwiches that are formed. **[2]**Since the Dunn et al. disclosure is directed to mapping RNA sequences this is deemed to be the target. **[3]**This target is located the same both in the Dunn et al. disclosure as well as in the practice of the instant invention and thus supports this rejection. **[4]**It is noted that the target in the Ranki et al. type of sandwich hybridization is in the middle of the sandwich and different from that of Dunn et al. as well as the instant invention.

D (i) Regarding assertions **[1]** and **[4]**, the Examiner is correct in his first statement that sandwich hybridization applies to both Dunn and Hassell (1977) and Ranki's U.S. Patent No. 4,486,539. The assertion that the location of the entity target is different between the two disclosures is incorrect, however. In both Dunn's and Ranki's disclosures, the target entity is clearly sandwiched between two probes having sequences which are complementary to different and nonoverlapping sequences in the target entity. In this regard, my reasons with respect to Dunn and Hassell (1977) have already been given in Paragraphs 7-12 above. With respect to the Ranki patent, see U.S. Patent No. 4,486,539, column 1, line 67, through column 2, line 16; see especially column 2, lines 6-16; see also Examples 1-4 and 6, and claim 1. A copy of Ranki et al., U.S. Patent No. 4,486,539 is attached as Exhibit 21.

D (ii) Regarding **[2]**, the Examiner's assertion is correct that the target in Dunn and Hassell (1977) is the RNA sequences to be mapped, my reasons and conclusions having being given in Paragraphs 8-12 above.

D (iii) Regarding **[3]**, the assertion that the target analyte in Dunn and Hassell (1977) is located the same as in the present invention is wrong. For reasons given above in Paragraphs 12A and 12B, the analyte in the present invention is altogether different from Dunn and Hassell (1977) both in its location as well as in its relationship to the molecular bridging and signaling entities.

14. For all of the reasons presented above, it is my opinion and conclusion that the present invention and pending claims define elements that are materially different from Dunn and Hassell (1977).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

March 24, 1998
Date

James G. Wetmur
James G. Wetmur, Ph.D

* * * * * * *

Enz-11(C2)(D1)(C2).Decl.JGW.3.23.98